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Short communication

Analyses of the putative Crp/Fnr family of transcriptional regulators of a serotype 4b strain of *Listeria monocytogenes* ☆

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Abstract

A whole-genome sequence analysis of *Listeria monocytogenes* strain F2365 revealed 15 potential members of the Crp/Fnr family of transcriptional regulatory proteins. Each gene and the flanking regions were cloned, subjected to in vitro transpositional mutagenesis, and recombined into strain F2365. Mutant strains, produced for 14 of the family members, were compared to strain F2365 for differences in carbon utilization, resistance to oxidative stress, and growth under reduced oxygen conditions that would signal an Fnr- or Crp-like function for these proteins. There were no differences among strain F2365 and the 14 mutant strains in the utilization of the carbon sources readily utilized by *L. monocytogenes*. Although strain KO2 had a reduced growth rate compared to strain F2365 and the other mutant strains at 30° but not at 37 °C, there were no differences in growth rates among strain F2365 and the mutant strains when incubated at either 30 or 37 °C under reduced oxygen conditions. However, when compared for differences in response to oxidative stress, mutants KO2 and KO5 showed reduced oxidative stress tolerance compared to the wild-type strain F2365. These results suggest that certain members of the putative Crp/Fnr family in *L. monocytogenes* may function in response to oxidative stress similar to the Fnr-like protein (Flp) of other Gram-positive bacteria.
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Keywords: *Listeria monocytogenes*; Fnr; Crp; Oxidative stress

1. Introduction

Listeria monocytogenes is a small rod-shaped bacterium found throughout the environment which can cause sporadic or epidemic cases of foodborne illness (Farber and Peterkin, 1991). Ingested bacteria invade gut epithelial cells and survive and spread in macrophages using a complement of well-described virulence genes which are under the control of the transcriptional regulatory protein PrfA, a member of the Crp/Fnr family of regulatory proteins (Leimeister-Wächter et al., 1990; Mengaud et al., 1991).

Advances in whole-genome sequencing and bioinformatics have produced a wealth of genomic information about several *Listeria* strains. A European consortium sequenced the complete genomes of *L. monocytogenes* strain EGD-e (serovar 1/2a) (GenBank/EMBL accession number AL591824) and of *L. innocua* strain CLIP 11262 (serovar 6a) (GenBank/EMBL accession number AL592022) (Glaser et al., 2001). More recently, we sequenced the genome of *L. monocytogenes* strain F2365 (serovar 4b, food isolate, 1985 Mexican-style cheese outbreak; (Linnan et al., 1988) and the results can be viewed on the TIGR web page at www.tigr.org (Nelson et al., 2004). Analyses of these genomes has revealed more than 2800 predicted protein-coding genes for each genome, and based on sequence homology, greater than

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30% of the putative genes have no predicted function. Many other genes have been classified into predicted classes or families, but remain functionally uncharacterized. Comparative sequence analyses of *L. monocytogenes* strains EGD-e and F2365 revealed that both strains contained 15 predicted members of the Crp/Fnr family of transcriptional regulators, including *prfA*. This is an unusually high number of Crp/Fnr sequences given that by comparison *Escherichia coli* has three members and *Bacillus subtilis* has only one member (Green et al., 2001).

The Crp and Fnr proteins of *E. coli* are founding members of a superfamily of structurally related transcriptional factors which are characterized by a sensory domain generally located in the N-terminus, a DNA-binding domain generally located in the C-terminus, and a long helix involved in dimer formation (Green et al., 2001). Members of this superfamily, both in Gram-negative and Gram-positive bacteria, are involved in regulation of a vast range of physiologic functions such as metabolism, anaerobic and aerobic respiration, resistance to oxidative stress, and virulence. Each of the 15 members of the Crp/Fnr-like regulators in *L. monocytogenes* has been assigned to this family based on protein similarities and motif analyses (Glaser et al., 2001).

The unusually high number of Crp/Fnr family members in *Listeria* as compared to other bacteria led us to question whether these regulatory homologues have a unique and important function for listeriae. We decided to comparatively investigate some basic functions of the entire Crp/Fnr family of *L. monocytogenes* strain F2365 using an in vitro mutagenesis system to produce isogenic mutants of each family member which could then be compared to the parent strain in a series of phenotypic screens. In this initial analysis of the group, we focused on determining if any of the family members showed differences in regulation of growth, respiration, oxidative stress tolerance, and/or carbohydrate metabolism which could indicate a functional homology to the previously described Crp- or Fnr-like proteins (Flp).

2. Materials and methods

2.1. Bacterial strains, growth media, and DNA techniques

L. monocytogenes strain F2365, a serotype 4b cheese isolate from the 1985 Mexican-style soft cheese outbreak in Los Angeles, California, was obtained from the Centers for Disease Control and Prevention (CDC, Atlanta, GA) (Linnan et al., 1988). *E. coli* strains JM109 (Promega Corp., Madison, Wisconsin) or TransforMax EC100 (Epicentre, Madison, Wisconsin) were used as hosts for all genetic manipulations in *E. coli,* except that *E. coli* Top10 (Invitrogen Corp., Carlsbad, California) was used as a host for PCR amplified products cloned into plasmid pCR2.1TOPO (Invitrogen). *L. monocytogenes* and *E. coli* strains were grown in brain–heart infusion (BHI; Difco Laboratories, Detroit, Michigan) broth. Pine's minimal media was used for testing *L. monocytogenes* mutant strains during reduced oxygen growth (Pine et al., 1989). Antibiotics (Sigma Chemical Co., St Louis, Missouri) were used at the following levels for *E. coli*: 100 μg/ml ampicillin, 50 μg/ml kanamycin, and 100 μg/ml spectinomycin. For *L. monocytogenes,* chloramphenicol was used at 10 μg/ml and spectinomycin was used at 50 μg/ml to maintain chromosomal transposon integrations. *L. monocytogenes* chromosomal DNA was obtained essentially as described by Flamm et al. (1984). Plasmid DNA was isolated from *E. coli* using Qiagen Spin Minipreps (Qiagen Inc., Valencia, California). Plasmid DNA was introduced into *L. monocytogenes* by electroporation (Park and Stewart, 1990).

2.2. Construction of L. monocytogenes mutant strains

Sequence strain data for *L. monocytogenes* strain F2365 are available on the TIGR web page at www.tigr.org. Chromosomal organization and features of the strain F2365 genome were viewed using the Sanger Centre informatics analysis software program, Artemis (Rutherford et al., 2000). Genome comparisons were performed using Artemis Comparison Tool (ACT) (Wellcome Trust Genome Campus, Cambridge, UK).

2.3. Construction of L. monocytogenes mutant strains

The entire open reading frame and 500–600 bp of flanking sequence were amplified for each of the 15 putative members of the Crp/Fnr family of *L. monocytogenes* using primers designed from the *L. monocytogenes* strain F2365 sequence database (www.tigr.org) and chromosomal DNA harvested from strain F2365 (Table 1). Amplified products were cloned into plasmid pCR2.1 TOPO to generate the recipient plasmids for in vitro mutagenesis using the GPS-Mutagenesis System (New England Biolabs, Inc., Beverly, Massachusetts). The donor plasmid, pGPS3 (New England Biolabs), was customized for use in *Listeria* by replacing the kanamycin resistance cassette with the spectinomycin cassette from plasmid pIC156 at the *Hin*dIII/*Not*I sites (Steinmetz and Richter, 1994). Mutagenesis was performed according to the manufacturer's protocol using BHI agar plates containing kanamycin and spectinomycin for recovery of recipient plasmids with transposon insertions. The orientation and location of transposon insertions within the recipient plasmid were determined by DNA sequencing using primers N and S (New England Biolabs) complementary to sequences on

Table 1
Crp/Fnr genes of *L. monocytogenes* strain F2365, primers used for amplification, and the transposon location following in vitro mutagenesis

Strain F2365 gene	Forward primer	Reverse primer	Transposon insertion[a]	Mutant strain name
LMOf2365_0130	tgtaggtaacatcactaaacagg	ttagcgaggccttcttagacg	Ω0130-158::Tn*7*[b]	MFS1384 (KO1)
LMOf2365_0171	catctgcgcgtccatcaagc	acaatcagtaaggcgcaagtgc	Ω0171-323:: Tn*7*[b]	MFS1366 (KO2)
LMOf2365_0294	gaaaaccttacgagctgacgtg	ggctatctattgaaacaacaggg	Ω0294-101:: Tn*7*[b]	MFS1367 (KO3)
LMOf2365_0301	cacacttcagaaggatcggaag	agacatcggacatgaaagcgtc	Ω0301-143:: Tn*7*[b]	MFS1368 (KO4)
LMOf2365_0577	cctaagtcttttgcgcgttgc	ggagacaatccttacattggtc	Ω0577-145:: Tn*7*[b]	MFS1369 (KO5)
LMOf2365_0626	ggttagctgcaaccattcttgg	tgtttctggctgggtactagc	Ω0626-437:: Tn*7*[b]	MFS1370 (KO6)
LMOf2365_0770	tttaccagtacctcgaacggtg	gcaccgattatccttataggagg	Ω0770-372:: Tn*7*[b]	MFS1371 (KO7)
LMOf2365_1265	tggcatgtataagtaagccatcg	cggtcaaggtttctatatgcgtc	Ω1265-237:: Tn*7*[b]	MFS1372 (KO8)
LMOf2365_2165	ctatcgaagcaaattgatctaacg	tgccagcagtgagagcgattc	Ω2165-66:: Tn*7*[b]	MFS1373 (KO9)
LMOf2365_2166	cgatgagtggcttcttacaagtg	aaggtgagctcgggactatcg	Ω2166-92:: Tn*7*[b]	MFS1374 (KO10)
LMOf2365_2197	ttggtctgtacatgggcagag	tccccgcttgactcagaatgg	Ω2197-441:: Tn*7*[b]	Not integrated
LMOf2365_2198	ttggtgctttagaagagattcgc	tacagacatctccaggttttagc	Ω2198-315:: Tn*7*[b]	MFS1375 (KO12)
LMOf2365_2731	caacaaggccggcaactgtag	gtatgttgcgccagctcgtgc	Ω2731-26:: Tn*7*[b]	MFS1376 (KO13)
LMOf2365_0777	cagaaagcatgtcgaaaccagc	ctgggaatgattggcatcaacg	Ω0777-382:: Tn*7*[b]	MFS1377 (KO14)
LMOf2365_0211 (*prfA*)	cattcacacctcgtcagtatgc	ctgaccatggtggtgttactcg	Ω0211-44:: Tn*7*[b]	MFS1378 (KO15)

[a]The transposon insertion site is defined as the number of nucleotides down-stream of the first nucleotide of the start-codon for each designated open-reading-frame (orf).
[b]Modified transposon Tn*7* (Sp^R).

opposing ends of the transprimer. Each of the 15 interrupted genes was moved into the multiple cloning site of integration vector pCON1, transformed into electrocompetent *L. monocytogenes* strain F2365, and selected for on BHI agar containing chloramphenicol and spectinomycin (Behari and Youngman, 1998). Chromosomal integration of the interrupted genes into *L. monocytogenes* strain F2365 was achieved as described (Freitag and Portnoy, 1994). Chromosomal integrations were verified by PCR amplification of product equivalent in size to the mutagenized gene bearing the spectinomycin resistant transprimer using forward and reverse nested primers (data not shown) designed from sequences outside of the mutagenized sequence. The correct integration and location of the transposon was verified by PCR using the nested primers in combination with the primers complementary to the transposon ends.

2.4. Biochemical substrate analysis

L. monocytogenes strain F2365 and each of the Crp/Fnr family mutants were tested for differences in metabolism of the "readily utilized" carbohydrates (i.e., glucose, fructose, mannose, *N*-acetylglucosamine, glucosamine, cellobiose, trehalose, maltose, and glycerol) using the carbon source Phenotypic MicroArrays according to the manufacturer's instructions (Biolog, Inc. Haywood, California) (Premaratne, et al., 1991). *N*-acetylmuramic acid which is not included on the carbon plates was not tested. Reactions were scored by visually comparing color changes in each well to the negative control well.

2.5. Growth studies

For aerobic growth studies, cultures grown in 2 ml BHI broth containing antibiotics as needed and incubated overnight with shaking were diluted 1:100 in 25 ml BHI broth in 250 ml flasks. Flasks were incubated in a shaking water bath at 130 rpm at the desired temperature and samples were taken at the designated time points for optical density readings at a wavelength of 600 nm. For the reduced oxygen growth curves, Pine's minimal media (30 ml) was dispensed into 30 ml glass screw-topped tubes and equilibrated in an anaerobic chamber (Bactron IV Anaerobic/Environmental Chamber, Sheldon Manufacturing, Inc., Cornelius, Oregon) for 96 h. The atmosphere within the chamber was maintained at 5% hydrogen, 10% oxygen and 85% nitrogen. Overnight cultures of each test strain grown in BHI with antibiotics as needed were inoculated 1:100 into the equilibrated Pine's media while inside the anaerobic chamber. The tubes were sealed, removed from the chamber, incubated at the appropriate temperature, and turbidity of the medium within the tubes was measured using a Biolog Turbidimeter (Model 21907, Biolog Inc.) at the designated time points.

2.6. Oxidative stress assays

Strains were tested for sensitivity to H_2O_2 as described (Wonderling et al., 2004). Briefly, overnight stationary-phase cultures of the wild-type and mutant strains, grown in BHI with antibiotics as needed, were diluted 1:100 in fresh BHI, and 150 μl of each dilution was spread onto BHI agar plates to generate a lawn. A 5-μl portion of sterile water containing 125 μg of H_2O_2

(Sigma) was added to 7 mm diameter disks cut from Whatman 3 mmChr chromatography paper (Whatman International Ltd, Maidstone, England) that were then placed on the spread plate. After overnight incubation at either 30 or 37 °C, the diameters of the zones of inhibition were measured in millimeters.

2.7. Statistical analyses

The absorbance or turbidity data collected from the growth studies in the initial screens were used to plot growth curves for each strain. An analysis of covariance was used to test the homogeneity of the calculated slopes and each strain was tested for differences in slope against the wild-type strain using SAS (SAS Institute Inc., Cary, North Carolina). The results are reported as doubling times which have an inverse relationship to the slope (Ingraham et al., 1983). Any strain, with a significantly different mean from that of strain F2365 during the screening test, was compared to the wild-type strain in three independent growth curves and the average means were tested using Student's *t*-tests. Strains were tested for resistance to oxidative stress using quadruplicate samples. The strains that showed differences from the wild-type strain were selected for additional study using 12 replicates per strain. The results, reported as the mean of measurements of the zones of inhibition in mm, were analysed using a one-way-analysis of variance and Dunnett's *t*-tests for means separation (SAS). Statistical significance for all comparisons was set at $P<0.05$.

3. Results and discussion

3.1. Genome analyses

The 15 predicted members of the Crp/Fnr family of strain F2365 are listed in Table 1 by their open-reading-frame (orf) designation from the most recent genomic sequence map (Nelson et al., 2004). The positioning of the Crp/Fnr-like genes with respect to adjacent genes in strain F2365 is identical to that determined for strain EGD-e. Comparison of the 15 members with each other and with family members from different organisms revealed little similarity in the protein sequences. Using BLAST 2 Sequences (www.ncbi.nlm.nih.gov/BLAST/bl2seq/), only 4 members (KO6, KO8, KO13, and KO14) of the strain F2365 Crp/Fnr family showed any sequence similarity to *E. coli* Crp, and none had greater than 22% identity. However, query of the Conserved Domain Database (www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi) identified COG0664, Crp, as either the highest scoring or the only hit for each of the 15 members, with 9e-24 as the highest E value (KO6). This suggests that motif analyses, rather than primary protein sequence identity, is more important for classification of the *L. monocytogenes* genes within the Crp/Fnr family.

Other than *prfA*, which is well characterized, features of the genome map indicate that 8 of the 14 members of the predicted Crp/Fnr family are not localized within an operon and would be transcribed independently. More specifically, the following 8 orfs: LMOf2365_0130, LMOf2365_0171, LMOf2365_0626, LMOf2365_0770, LMOf2365_1265, LMOf2365_2165, LMOf2365_2166, and LMOf2365_2731 are each followed by a predicted transcriptional terminator or a convergent downstream gene or both. Of the remaining 6 orfs, LMOf2365_0294 and LMOf2365_0301 are terminal members of potential 3-gene (no predicted function) and 4-gene (weak homology to an ABC transporter) operons, respectively. Both are followed by predicted transcriptional terminators and convergent downstream genes.

The LMOf2365_2198 and LMOf2365_2197 orfs are positioned adjacent to each other, and transcribed in succession, with orf LMOf2365_2197 following LMOf2365_2198. Predicted transcriptional terminators precede and follow the gene pair, but there is no predicted termination site between the genes, thus making it possible that disruption of LMOf2365_2198 could have polar affects on LMOf2365_2197. Also, LMOf2365_0577 and LMOf2365_0777 are part of 5 and 2 gene operons, respectively, and their disruption could cause polar affects on downstream genes: LMOf2365_0577 is followed by a gene with weak homology to an internalin and LMOf2365_0777 is followed by an orf similar to a bile acid 7-α-hydratase protein.

As most of the genes within this family are positioned such that their disruption would be unlikely to exert polar affects on neighboring genes, we chose a transposon-based system to generate insertional mutants for each family member. Any identified phenotypes for genes with expected polar affects can be subsequently tested using complementation analyses. The location of transposon insertions in each mutant strain and their respective knock-out (KO) strain designations are shown in Table 1. Fourteen of the mutanted genes were successfully integrated into the chromosome of strain F2365 as verified by PCR, generating 14 stable, mutant strains. Chromosomal integration of the mutated LMOf2365_2197 was attempted multiple times, but it was the only member of this predicted gene family whose mutant allele could not be recombined back into the genome. This could signify an essential function for the product of LMOf2365_2197.

3.2. Carbohydrate utilization

To test for regulatory affects similar to *E. coli* CRP, strain F2365 and each of the 14 Crp/Fnr mutants were

tested for differences in metabolism of the "readily utilized" carbohydrates for *L. monocytogenes* as described by Premaratne et al. (1991). In two separate studies, the wild-type and mutant strains each utilized all 9 carbohydrates. This suggests that, unlike Crp in certain Gram-negative bacteria, the Crp/Fnr homologues of strain F2365 do not function in *Listera* as major regulators of carbohydrate utilization (Saier et al., 1995). This may not be surprising in the light of research which indicates that catabolite repression in Gram-positive bacteria is not mediated by a Crp-like protein, but rather through proteins like CcpA and HPr acting with CRE (catabolite repression element) sequences (Saier et al., 1995). Further studies will be required to fully define any metabolic differences among the mutant strains described herein.

3.3. Growth analysis

To test the mutant strains for any Fnr-like affects, we measured the growth rates of the 14 mutants and strain F2365 under reduced oxygen conditions at both 30 and 37 °C in Pine's Minimal Media. Under reduced O_2 conditions, the doubling times for strain F2365 and the 14 mutant strains ranged from 120.1 to 151.9 min at 37 °C and from 355.5 to 599.9 min at 30 °C. There were no statistical growth differences for any strains compared to the wild type at either growth temperature. These findings suggest that, unlike *E. coli* Fnr, none of the mutated genes has a major role in anaerobic growth. However, more studies identifying the favored *L. monocytogenes* electron acceptors and limiting their concentration in the growth media under reduced O_2 conditions should be conducted to fully investigate any role for anaerobic growth.

We also screened the growth rates of the 14 mutant strains and strain F2365 in BHI broth at temperatures of 37, 30, and 4 °C in the presence of oxygen. When tested at 4 °C in the presence of oxygen, the wild-type strain and the mutants had doubling times that ranged from 0.93 to 1.33 d and showed no statistical differences in growth compared to the wild-type strain. This suggests that none of the predicted 14 Crp/Fnr genes are required for adaptation of *L. monocytogenes* F2365 to cold temperatures in the presence of oxygen. At 30 °C, growth of mutant strain KO2 in the presence of oxygen was significantly less than that of strain F2365. The doubling times for strain F2365 and all of the mutant strains ranged from 65.4 to 92.8 min and no strains were statistically different from strain F2365 at a 0.05 level of significance except strain KO2 (doubling time = 121.0 min, $P = 0.024$). At 37 °C in the presence of oxygen, the doubling times for these same 14 strains ranged from 52.1 to 81.8 min, except for strain KO2 which had a doubling time of 100.7 min. Although none of the strains showed a statistically significant growth difference when compared to strain F2365 at 37 °C, strain KO2 approached the significant level ($P = 0.058$).

We further explored potential differences between strains F2365 and KO2 by comparing the average doubling times from three independent growth studies of both strains at 30 and 37 °C in the presence of oxygen and tested the average means using Student's *t*-tests (Fig. 1). Again, mutant strain KO2 [mean doubling time (MDT) = 74.5 min, mean standard deviation (MSD) = 1.2 min] grew significantly slower than the wild type (MDT = 70.8 min, MSD = 1.7 min) at 30 °C. However, the growth of strain KO2 (MDT = 68.8 min, MSD = 6.3 min) and strain F2365 (MDT = 69.9 min, MSD = 5.0 min) were not significantly different at 37 °C. These data indicate that the expression of LMOf2365_0171 (KO2) is required for maximal growth in rich media at 30 °C.

3.4. Oxidative stress assays

The Flp of *Lactobacillis casei* and *Lactococcus lactis* function in the recognition of and resistance to oxidative stress (Gostick et al., 1998; Gostick et al., 1999). We tested the *L. monocytogenes* Flp for a similar function by comparing strain F2365 and the 14 mutant strains for sensitivity to H_2O_2 (Table 2). Mutants KO2 and KO5 were significantly more sensitive to H_2O_2 challenge than

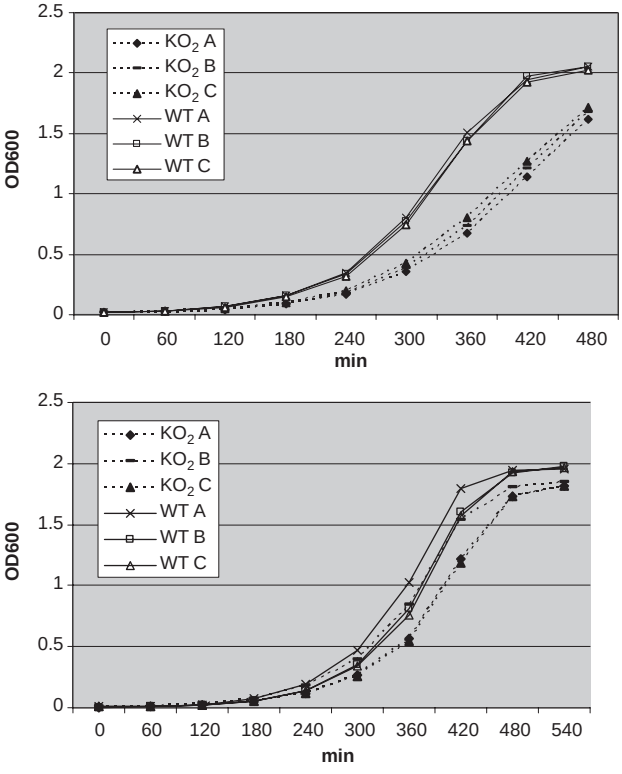

Fig. 1. Growth rate comparison of *L. monocytogenes* strain F2365 (WT) and mutant strain KO2 grown aerobically in BHI broth at 30 °C (top panel) and 37 °C (bottom panel) in each of three (A, B, C) trials.

Table 2
Results of oxidative stress assays for selected (pre-screened) Crp/Fnr mutants and strain F2365 at 30 and 37 °C

Strain	H_2O_2 inhibition average zone diameter in mm at 30 °C	H_2O_2 inhibition average zone diameter in mm at 37 °C
F2365	17.5	18.9
KO 2	22.9[a]	22.9[a]
KO 3	[b]	[b]
KO 4	[b]	[b]
KO 5	19.8[c]	20.7[c]
KO 6	18.0	19.1
KO 7	18.7	19.0
KO 8	18.5	20.2
KO 9	18.8	20.0
KO 10	[b]	[b]
KO 12	18.6	20.5[c]
KO 13	19.1[c]	20.3
KO 14	[b]	[b]
KO 15	17.6	20.0

The values are measurements of the zones of inhibition around H_2O_2 impregnated disks.
[a] $P<0.05$ vs. all other strains as determined by the Dunnett's *t*-tests.
[b] No differences vs. strain F2365 detected on the initial screen.
[c] $P<0.05$ vs. strain F2365 as determined by the Dunnett's *t*-tests.

the wild-type strain during incubation at both 30 and 37 °C, with KO2 being more sensitive than KO5. During incubation at 30 °C strain KO13 was more sensitive than the wild type and at 37 °C mutant KO12 was also more sensitive than the wild-type strain F2365. These results indicate that both LMOf2365_0171 (K02) and LMOf2365_0577 (K05) are involved in the oxidative stress response, with LMOf2365_0171 (KO2) required for maximal resistance. The *flpA* and *flpB* genes of *L. lactis* are involved in resistance to oxidative stress, which may be mediated through regulation of intracellular zinc levels (Gostick et al., 1999; Green et al., 2001). Interestingly, LMOf2365_0171 (K02) is positioned adjacent to an operon with homology to a zinc uptake system.

Although the currently sequenced strains of *L. monocytogenes* contain a large number of orfs with sequence and motif similarities to the Crp/Fnr family of genes, there have been no previous analyses of these potential regulatory genes. Our results suggest that certain members of the Fnr- and Crp-like family proteins of *L. monocytogenes* strain F2365 may function to monitor and control the response to environmental oxidative stresses similar to the role of the Flp genes of *Lactobacillus*. However, none of these 14 *L. monocytogenes* genes had an affect on growth under aerobic or anaerobic growth conditions nor did they mediate catabolite repression of the readily utilized carbohydrate sources. A more detailed analysis of the potential regulatory affects of these genes, as well as their role in relation to food safety, will be conducted on an individual basis.

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